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Co-expression of slow-twitch/cardiac muscle Ca^{2+}-ATPase (SERCA2) and phospholamban

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Full length cDNAs encoding both slow-twitch/cardiac (SERCA2) and fast-twitch skeletal muscle (SERCA1) Ca^{2+}-ATPases were expressed by transient transfection of COS-1 cells. Studies of the Ca^{2+}-dependency of Ca^{2+}-transport in microsomes isolated from these cells showed that both isoforms had an affinity for Ca^{2+} of about 0.2 μM. The Ca^{2+}-affinity of SERCA2 was lowered when phospholamban was co-expressed with it, demonstrating that the two proteins interact in this expression system. These studies support the view that phospholamban inhibition accounts for the low Ca^{2+}-affinity and low activity of SERCA2 in cardiac muscle sarcoplasmic reticulum.

Ca^{2+}-ATPase; Phospholamban; Co-expression; Ca^{2+}-affinity

1. INTRODUCTION

Ca^{2+}-ATPases play a crucial role in muscle relaxation through their ability to pump Ca^{2+} out of the sarcoplasm [1,2]. At least 3 different Ca^{2+}-ATPase genes are expressed in mammalian cells [3–7]. One isoform, SERCA1, is expressed almost exclusively in fast-twitch skeletal muscle [4,5], while SERCA2, and to a minor extent, SERCA3, are expressed in cardiac and slow-twitch skeletal muscles [3,5,7]. Thus most of the Ca^{2+}-transport activity of cardiac muscle sarcoplasmic reticulum can be ascribed to the SERCA2 isoform. The Ca^{2+}-pump in cardiac sarcoplasmic reticulum vesicles has a lower affinity for Ca^{2+} than does the Ca^{2+}-pump in fast-twitch skeletal muscle sarcoplasmic reticulum [8]. It is not clear whether the low affinity for Ca^{2+} of SERCA2 in cardiac sarcoplasmic reticulum can be explained entirely by the presence of the inhibitory protein, phospholamban [2], or whether it is due to amino acid sequence differences, since the amino acid sequence identity is only 76% between the two isoforms [4].

In this communication, we demonstrate that the two isoforms expressed in isolation of other sarcoplasmic reticulum proteins show almost the same Ca^{2+}-affinity, indicating that other components, including phospholamban, must be involved in suppression of the Ca^{2+}-affinity of the Ca^{2+}-ATPase in cardiac muscle sarcoplasmic reticulum. We also demonstrate that co-expression of SERCA2 with phospholamban lowers the affinity of the pump for Ca^{2+}.

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2. MATERIALS AND METHODS

The cDNAs encoding the full length rabbit SERCA2 [3] and SERCA1 [4] cloned into the mammalian expression vector pcDX [9] were utilized for transfection of COS-1 cells. The same cDNA inserts ligated into the expression vectors pRK1-4 and p91023(B) [10,11] were also utilized. The construct for expression of rabbit phospholamban cDNA in p91023(B) was reported previously [12]. In order to assure co-expression of rabbit SERCA2 and phospholamban in the same cell, cDNAs encoding both SERCA2 and phospholamban cDNA were incorporated into one plasmid vector under the regulation of separate promoters. The expression vector pSVL (Pharmacia) was cleaved at unique *Eco*RI and *Sal*I sites and a fragment containing the multiple cloning site and the elements necessary for cDNA expression in mammalian cells, including the SV40 origin of replication, was isolated. This fragment was blunt-ended and ligated with *Sal*I linkers. After digestion with *Sal*I, the fragment was ligated into the unique *Sal*I site in the pcDX vector containing full length SERCA2 cDNA [3], upstream of the regulatory sequences controlling expression of the ATPase. The rabbit phospholamban cDNA fragment, fitted with *Sst*I linkers [12], was then ligated into the unique *Sst*I site in the pSVL-derived multiple cloning sequence. The same construct containing SERCA2 cDNA in the correct orientation and phospholamban cDNA in the reverse orientation was utilized as a control for analysis of the effect of phospholamban on ATPase function. The construct was amplified and purified by CsCl-gradient centrifugation.

COS-1 cells were transfected by the DEAE-dextran method [13] using 15 μg of the construct per 10 cm dish. Cells were harvested after 48 or 72 h and the microsomal fraction was prepared and utilized for Ca^{2+}-uptake measurement and for immunoblotting as described

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previously [11]. A monoclonal antibody A1, raised against dog phospholamban [14] and a polyclonal antibody C4, raised against a fusion protein containing the NH_2-terminal one-fifth of the rabbit SERCA2 [6] were utilized for immunostaining to demonstrate expression of phospholamban and the Ca^{2+}-ATPase, respectively.

3. RESULTS AND DISCUSSION

In a previous publication [11] we described the expression of the rabbit SERCA1 protein and measurement of Ca^{2+}-uptake activity by microsomes isolated from cDNA-transfected COS-1 cells. In this study, we show that SERCA2 can also be expressed in a functional form. Fig. 1A indicates the time course of Ca^{2+}-uptake by the microsomes from cells transfected with SERCA1 and SERCA2 cDNAs. Ca^{2+}-transport rates were linear for at least 20 min. When the Ca^{2+}-dependency of Ca^{2+}-transport was compared between the two isoforms by varying free Ca^{2+}-concentrations in the assay media (Fig. 1B), the Ca^{2+}-concentration required for half-maximal activity was found to be 0.1–0.2 μM in both cases. Since the Ca^{2+}-affinity of the pump in cardiac sarcoplasmic reticulum has been reported to be 3–6-fold lower than that in fast-twitch skeletal muscle sarcoplasmic reticulum [8], but the pumps have nearly the same Ca^{2+}-affinity in isolation, a component must exist in cardiac sarcoplasmic reticulum which alters the affinity of SERCA2 for Ca^{2+}, thereby inhibiting its activity in low free Ca^{2+}-concentrations. Dephospho-phospholamban has been shown to be an inhibitor of SERCA2, but phosphorylation of phospholamban relieves this inhibitory effect [15].

In order to investigate the effect of phospholamban on Ca^{2+}-uptake rates, we made an expression construct in which both SERCA2 and phospholamban cDNAs were ligated into one plasmic vector, but with separate promoters. As shown in Fig. 2, the cells transfected with the plasmic containing these cDNAs expressed both proteins. Phospholamban synthesis was detected with the monoclonal antibody A1. Antibody C4 detected not only the transfected Ca^{2+}-ATPase, expressed as a result of transfection, but also the low level of endogenous Ca^{2+}-ATPase in COS-1 cell microsomes [6]. SERCA2 expressed together with phospholamban had a lower affinity for Ca^{2+} than SERCA2 expressed

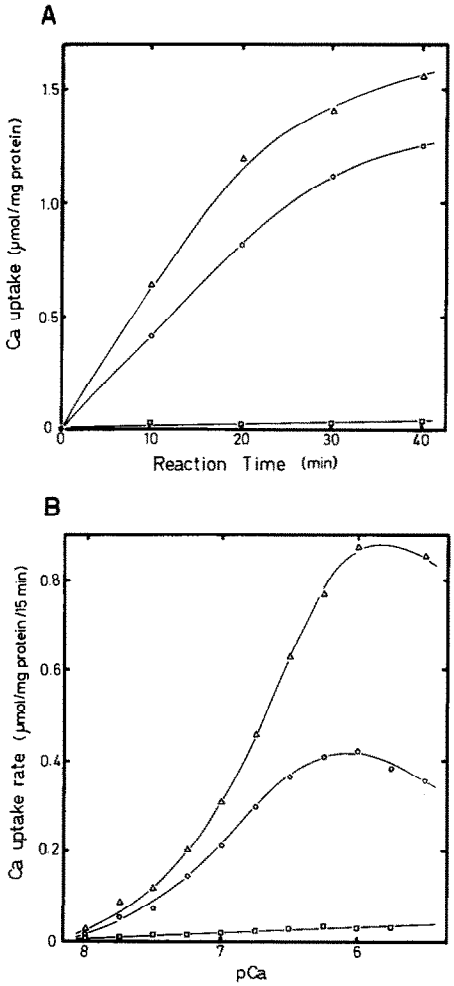

Fig. 1. Ca^{2+}-uptake by microsomes transfected with fast- (SERCA1) and slow-twitch/cardiac (SERCA2) muscle Ca^{2+}-ATPase cDNAs. (A) Time course of Ca^{2+}-uptake at 2 μM free Ca^{2+} and; (B) Ca^{2+}-dependency of Ca^{2+}-transport at a fixed time of 15 min, in the presence of various concentrations of free Ca^{2+} by SERCA1 (▲), SERCA2 (○) and control microsomes (□). The experiments described in A and B were carried out with different preparations with different levels of expression of the Ca^{2+}-ATPase, accounting for the differences in specific activities between the two experiments.

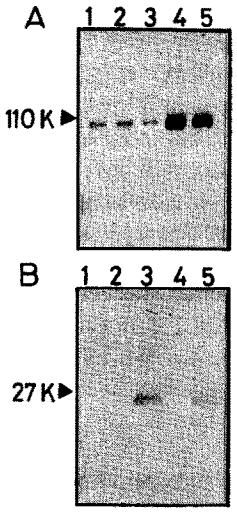

Fig. 2. Immunoblotting of microsomal proteins. 25 μg of microsomal proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 8% (A) and 12.5% (B) gels, and transferred to nitrocellulose membranes. Polyclonal antibody C4, (A) and monoclonal antibody A1, (B) were utilized to detect SERCA2 (110 kDa) and phospholamban (27 kDa) expression, respectively. The microsomes are: lane 1, control; lane 2, transfected with vector only; lane 3, transfected with phospholamban cDNA; lane 4, transfected with SERCA2 cDNA; and lane 5, transfected with SERCA2 plus phospholamban cDNAs.

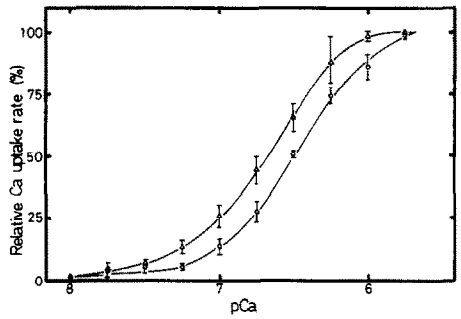

Fig. 3. Ca^{2+}-dependency of Ca^{2+}-transport by microsomes expressing Ca^{2+}-ATPase alone (▵) and Ca^{2+}-ATPase plus phospholamban (○). The relative Ca^{2+}-uptake rates measured at a fixed time of 15 min are shown, with the maximum Ca^{2+}-uptake rates equal to 100%. Each data point represents the mean ± the standard deviation for triplicate experiments with different microsomal preparations.

alone (Fig. 3). The difference in affinity that could be attributed to co-expression with phospholamban was about 0.2–0.3 pCa units. This corresponds to the Ca^{2+}-affinity in the native cardiac sarcoplasmic reticulum preparation, where the difference in Ca^{2+}-affinity attributable to phospholamban phosphorylation is about 0.3 pCa units [16]. Thus it is clear not only that SERCA2 and SERCA1 have the same intrinsic Ca^{2+}-affinities but also that phospholamban alone is capable of altering the Ca^{2+}-affinity of SERCA2. We also found that COS-1 cells cotransfected with SERCA1 and phospholamban cDNAs displayed lower affinity for Ca^{2+} than that of SERCA1 alone, similar to the results presented in Fig. 3 (not shown).

Phospholamban seems to exert its effect on SERCA2 through ionic interactions in the cytoplasmic domains of the two proteins [17,18]. Kim et al. [19] demonstrated an inhibitory interaction between SERCA2 and a peptide consisting of residues 1–25 of the cytoplasmic domain of phospholamban. They found that a high molar ratio between the synthetic phospholamban peptide and the Ca^{2+}-ATPase protein was necessary to inhibit Ca^{2+}-uptake activity in reconstituted vesicles, however. Since phospholamban forms a pentamer [20,21] and the intramembranous domain is responsible for pentamer formation [12], the pentameric form may have a stronger interaction with the Ca^{2+}-ATPase than the monomeric form. Although the stoichiometry is not clear, the most likely in vivo ratio is one phospholamban monomer per Ca^{2+}-ATPase molecule [22].

It is possible that higher phospholamban expression might be necessary in our system to reproduce the same high inhibitory effect as that found in the native cardiac sarcoplasmic reticulum. No significant change in the Ca^{2+}-dependency of Ca^{2+}-transport was observed, however, when an equimolar ratio of phospholamban cDNA in the p91023(b) vector [12] was transfected together with the modified pcDX expression vector containing both SERCA2 and phospholamban cDNAs. Under these conditions, the ratio between phospholamban and ATPase expression was increased, although total ATPase synthesis was decreased. An additional factor might be that the microenvironment of the endoplasmic reticulum from COS-1 cells might differ from that of the sarcoplasmic reticulum, leading to changes in phospholamban function. For example, Suzuki and Wang [23] have shown that phosphatidylinositol is important for maximal phosphorylation of phospholamban.

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